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RADIOBIOLOGIC RESPONSE OF CHO–KI CELLS TREATED WITH VITAMIN A

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Abstract

Treatment of CHO–KI cells with vitamin A altered their response to subsequent gamma irradiation. In general longterm preincubation with low doses of the vitamin caused a relative increase in the number of cells surviving a given radiation dose. The effect resulted in an increase in the D_0 of the survival curve. Long or short term exposure to high concentrations of the vitamin caused a decrease in the number of surviving cells leading to a decrease in the extrapolation number of the survival curve. Recovery of cells from radiation damage, assessed using the split dose technique, was also impaired by vitamin A pretreatment. A mechanism involving repair of potentially lethal damage may explain the protective effect of vitamin A since this was highly dependent on the cell density of cultures at the time of irradiation. However, in view of the data showing that the vitamin A concentrations necessary to alter the radiation survival curve shoulder caused a significant release of sialic acid into the medium, a mechanism involving membrane stability may account for both the reduction in repair/recovery capacity of the treated cells and the radioprotective effect.

Key words: Radiation biology, cell studies, vitamin A.

Recent evidence suggests that a study of the effects of vitamin A on the response of cells to ionising radiation may be of both theoretical and practical interest. The use of the vitamin and its synthetic analogues (retinoids) in epithelial tumour treatment is undergoing extensive investigation at present (8, 12, 29). Since many epithelial tumours are treated with irradiation, we thought it of interest to look for interactions between the two agents, following indications that the vitamin alters the effect of radiation (4, 20). The interaction may also be of theoretical interest in attempts to elucidate mechanisms of radiation damage and repair/recovery in cells, since the vitamin is known to have a general effect on cell membranes (17), particularly those of mitochondria (25) and lysosomes (6) and probably through membrane related mechanisms alters energy metabolism in the cell (25). There is increasing evidence that both membrane related phenomena and energy metabolism are involved in the cell's response to irradiation (1, 7, 10, 27).

In the present study, the effect of vitamin A on the radiation response of Chinese Hamster Ovary (CHO)-KI cells to ^{60}Co gamma irradiation was investigated under a variety of conditions.

Material and Methods

Cell culture. CHO–KI cells (Flow Laboratories, Scotland) were grown in Ham's F12 nutrient mixture supplemented with 10% foetal calf serum. They were subcultured twice weekly using standard procedures and kept in exponential growth phase. For most experiments about 5×10^4 cells were seeded in 5 ml growth medium in sealed 40 ml flasks (Nunc, Denmark) with a 25 cm^2 growth area. The cell number normally increased over the experimental period to reach 4×10^6 cells at the time of irradiation. This level has been shown to be subconfluent under the experimental conditions used in this report (26). After 3 days growth the medium was changed on all flasks and replaced with either ordinary medium, medium containing 0.4% ethanol (which was found to be non-toxic) or medium containing appropriate concentrations of vitamin A in 0.4% ethanol. Just before irradiation the medium in all flasks was changed again and the flasks were irradiated immediately. Where the effect of the time of exposure to the vitamin was being examined, the medium was changed 24 hours before irradiation and then 0.5 ml of medium containing 10 times the desired final concentration of vitamin/solvent was added at appropriate times before irradiation to 4.5 ml medium already in the test flasks with as little disturbance of the cells as possible.

Three hours after irradiation the cells were rapidly trypsinised, diluted and plated in fresh medium in Petri dishes.

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Cell survival was assessed 6 days later using the colony formation technique (22). Plating efficiency of control and solvent treated cultures was approximately 70% under these conditions.

Vitamin A uptake studies. Levels of the vitamin taken up by the cells were estimated using the Carr-Price method (14). Cells were first trypsinised and the suspension centrifuged at 3000 rpm for 10 min; the resulting pellet was washed twice in Earle's Balanced Salt Solution before being taken up in 2 ml absolute ethanol. Four ml petroleum ether (bp. 40–60°C) was added and the mixture shaken for 10 min. The layers were allowed to separate, after which the petroleum ether layer was removed and evaporated in a stream of nitrogen. The residue was taken up in 0.5 ml chloroform and placed in a cuvette in the spectrophotometer set at 620 nm. Two ml antimony trichloride was added and a reading taken within 10 s. The level of vitamin A was then read off a standard graph.

Measurement of sialic acid release. Sialic levels were measured in medium samples from vitamin A experiments using BCL Kit No. 784192 where n-acetyl-neuramine acid (NANA) is converted to N-acetyl mannosamine and pyruvate in the presence of NANA-aldolase. The pyruvate is oxidised in the presence of flavin adenine nucleotide (FAD) and thiamine pyrophosphate to acetyl phosphate, CO_2 and H_2O_2 in the presence of pyruvate oxidase. The amount of H_2O_2 formed, which is equivalent to the free NANA is converted by peroxidase in the presence of 4-aminoantipyrine and N-ethyl-N-2-hydroxyethyl-3-tolduidine to a red dye. The absorbance of this is measured at 550 nm.

Irradiation. Cells were irradiated using a ^{60}Co therapy unit which delivered 1.2 Gy/min at 70 cm SSD (skin source distance).

Statistical analysis. All experiments were repeated at least 3 times. Results are the mean ± standard error of the pooled results. Within each experiment, cells from 3 replicate flasks were each plated into 3 Petri dishes giving a total of 9 points/experiment and 27 points/final result. Survival curve parameters (n and D_0) were determined using linear regression analysis of the exponential part of the curve.

Results

Uptake of vitamin A by cells. Fig. 1 shows the levels of vitamin A detected in washed cell pellets following the treatments used in the experiments. In Fig. 1a the levels detected following varying lengths of exposure to a level of 15 μg (vitamin A)/ml are shown; a rapid initial uptake complete in less than 10 min is followed by a slower rate of uptake which continues up to 24 h. In Fig. 1b the levels detected in cells exposed to varying concentrations of vitamin A, from 0–40 μg/ml, for 24 h are shown. The vitamin A content of the cells can be seen to increase with the level in the medium.

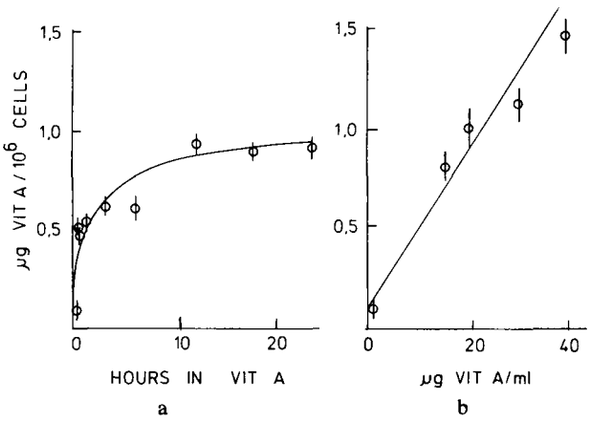

Fig. 1. Uptake of vitamin A by CHO–KI cells a) as a function of time of exposure to a medium concentration of 15 μg/ml and b) exposed to increasing concentrations of the vitamin over a 24-h period.

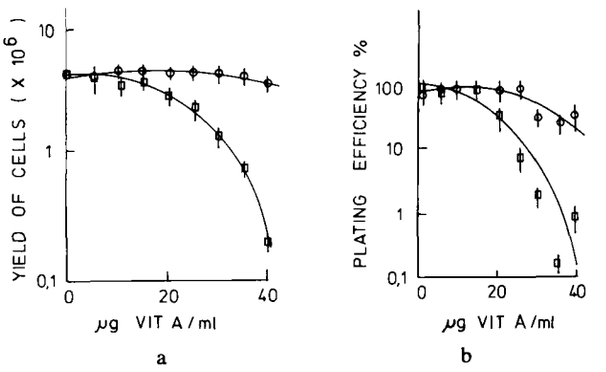

Fig. 2. Effect of exposure to increasing concentrations of vitamin A on a) the yield and b) subsequent plating efficiency of CHO–KI cells. 3-h exposure (○). 24-h exposure (□).

Cell growth and subsequent plating efficiency in vitamin A treated cultures. Exposure of cells to increasing levels of vitamin A for 24 h gradually reduces their yield at concentrations up to 20 μg/ml, a severe exponential toxic effect can be detected at vitamin A concentrations above 20 μg/ml (Fig. 2a). The subsequent plating efficiency of treated cells is similarly affected (Fig. 2b). Treatment of cells for 3 h had no effect on the yield of cells but their subsequent plating efficiency was reduced at the high vitamin A levels (>30 μg/ml).

Radiation response of cells following treatment with vitamin A. Incubation of cells in low levels of vitamin A for 24 h before irradiation to 6 Gy was found to induce radioprotection. Higher levels induced a reduction in survival (Fig. 3). Vitamin A was present during irradiation and 3 h after irradiation for these experiments. The exact vitamin A level at which the overall radiation response changed from protection to reduction in survival was found to depend on the radiation dose (Fig. 4). The figure

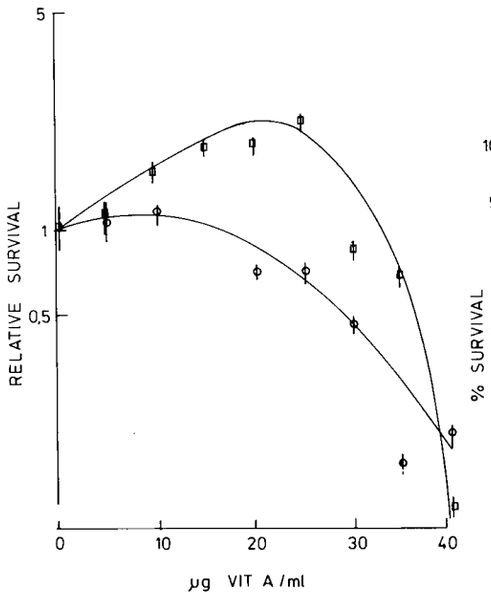

Fig. 3

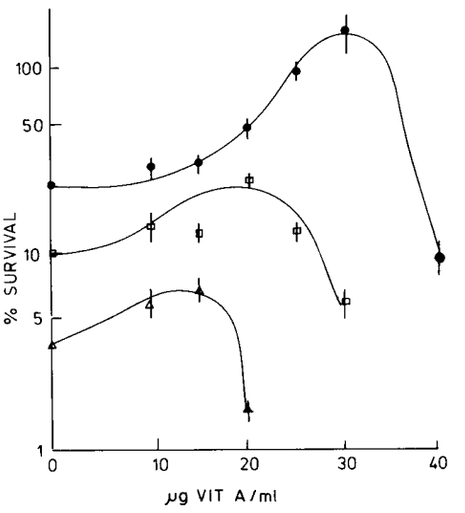

Fig. 4

Fig. 3. Survival of vitamin A treated CHO–KI cells exposed to 6 Gy gamma rays. 24-h pre-exposure to the vitamin (□). Vitamin A present during irradiation and for 3 h after irradiation (○).

Fig. 4. Survival of CHO–KI cells exposed to increasing levels of vitamin A for 24 h before 5 Gy (○), 7.5 Gy (□) and 10 Gy (△).

Fig. 5. Effect of time of exposure of CHO–KI cells to vitamin A on the survival of cells subsequently irradiated to 10 Gy. Vitamin A (15 μg/ml) (□). Ethanol (solvent control) (△). Control (○).

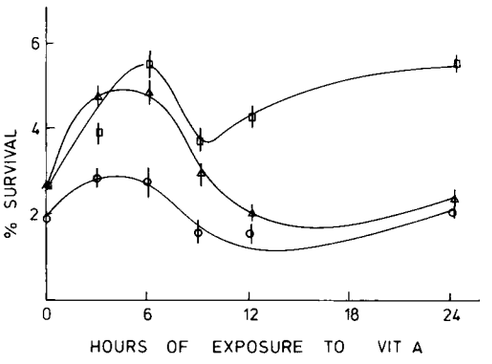

Fig. 5

shows that if cells are treated with a range of vitamin A levels (0–40 μg) for 24 h and then irradiated to 5, 7.5 or 10 Gy the maximum radioprotective effect shifts from a vitamin A concentration of 15 μg/ml vitamin A at 10 Gy through ~20 μg/ml at 7.5 Gy to 30–35 μg/ml at 5 Gy.

The effect of time of exposure to vitamin A on the radiation response. Experiments designed to ascertain the duration of vitamin A pretreatment necessary to produce a radioprotective effect (Fig. 5) indicate that when using a low level of the vitamin (15 μg/ml) no net protective effect can be detected unless it has been in contact with the cells for at least 12 h. The transitory increase in survival seen where vitamin A is added 3 to 6 h before irradiation is also seen with the ethanol solvent alone and even where ordinary medium was added. It is, therefore, deemed to be due to processes others than those involving vitamin A.

The effect of vitamin A on radiation survival curves. Survival curves obtained following 24 h pretreatment using a range of vitamin A levels which produce radioprotection (10–20 μg/ml) reveal that in this concentration range the protective effect is associated with an increase in the D_0 of the survival curve (Fig. 6).

The levels of the vitamin needed to cause a significant reduction in radiation survival are associated with high levels of cytotoxicity. Since this makes interpretation of survival curves difficult due to the high cell numbers required for cloning experiments, the effect of high levels of the vitamin was examined only following short time periods of exposure. Treatment with 40 μg vitamin A/ml for 3 h produced a reduction in D_0 from 1.7 to 1.3 and a severe effect on the extrapolation number, which fell from 6.8 to 2.7 (Fig. 7). Short times of exposure to lower levels of the vitamin (10–20 μg/ml) had no significant effect. n and D_0 values for both sets of survival curves are presented in Table 1. These confirm that the radioprotective effect is reflected in an increase in the D_0 of the survival

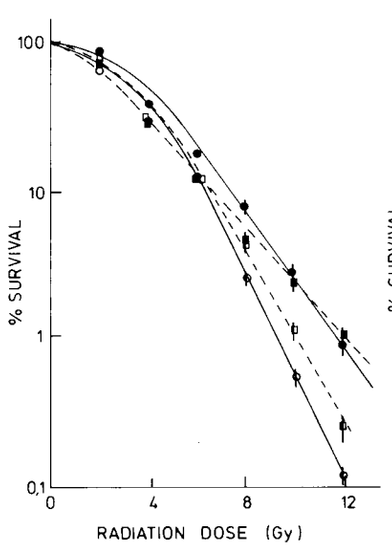

Fig. 6

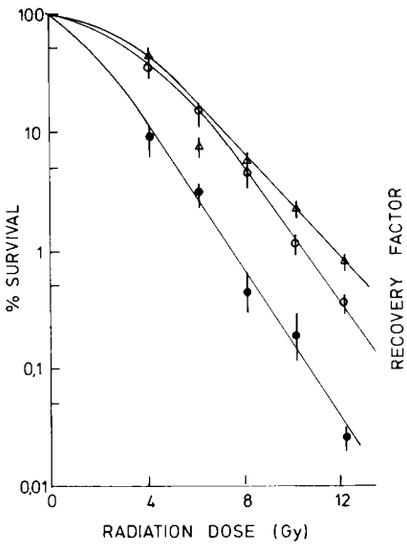

Fig. 7

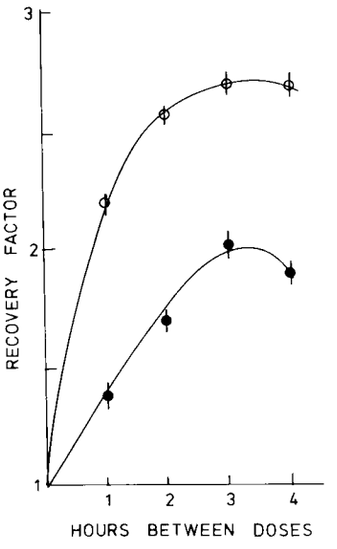

Fig. 8

Fig. 6. Survival curves for CHO–KI cells exposed to vitamin A for 24 h before irradiation. Control (○). 10 μg/ml (□), 15 μg/ml (●) and 20 μg/ml (■) vitamin A.

Fig. 7. Survival curves for CHO–KI cells exposed to vitamin A during irradiation and for 3 h afterwards. Solvent control (○). 30 μg/ml (△) and 40 μg/ml (●) vitamin A.

Fig. 8. Recovery curves for CHO–KI cells exposed to vitamin A for 24 h before irradiation. Results plotted as relative survival of cells exposed to 5 Gy initially, followed by 5 Gy 0 to 4 h later. Control (○). 20 μg/ml vitamin A (●).

curve following prolonged exposure, while high levels of the vitamin for short periods reduce the extrapolation number.

The effect of vitamin A on split dose recovery. Split dose recovery experiments, where cells pretreated for 24 h with 20 μg/ml vitamin A were given 5.0 Gy as a conditioning dose and a further 5.0 Gy 1 to 4 h later, showed that vitamin A pretreatment reduced the final relative recovery factor of cells from 2.76 to about 2.0. The rate of recovery over the first 3 h was also altered (Fig. 8).

The effect of cell number on the radiation response following vitamin A treatment. Cell cultures with densities ranging from 0.28×10^6 to 5.7×10^6 were irradiated to 10 Gy following 24 h treatment with 15 μg vitamin A/ml. The results (Table 2a) show that the radioprotective effect of vitamin A is greatest in high density cultures. In another series of experiments (Table 3) cultures seeded with cloning densities and irradiated to 10 Gy showed a reduction in survival at every vitamin A level tested.

Sialic acid release following vitamin A treatment. Table 4 shows the results of experiments where cells were exposed to various levels of vitamin A for 3 or 24 h after which the sialic acid release into the medium was measured. The results indicate that exposure to vitamin A increases the level of sialic acid release. The effect is highest following short times of exposure and is not markedly dependent on the concentration of the vitamin.

Table 1

The effect of vitamin A treatment on the survival curve parameters n and D_0, calculated from regression lines drawn to the data in Figs 6 and 7

Vitamin A concentration (μg/ml)	24-hour exposure		3-hour exposure	
	n	D_0	n	D_0
0	7.4	1.4		
10	7.5	1.7	6.8±0.5	1.7±0.18
15	4.0	2.0		
20	2.0	2.5		
30	–	–	3.21	12.0
40	–	–	2.68	1.3

Pooled data: No significant difference between values for 3 hours exposure to 0–20 μg/ml vitamin A.

Table 2

The relationship between cell number and degree of radioprotection for cells treated with 15 μg/ml vitamin A for 24 hours prior to 10 Gy

Cell No. at time of irradiation	Protective factor
5.71×10^6	4.69
2.48×10^6	2.85
0.44×10^6	1.78
0.28×10^6	1.63

Table 3

The effect of increasing levels of vitamin A on radiation response of microcolonies irradiated to 10 Gy following 24 hours treatment with 0–15 μg/ml vitamin A

Vitamin A (μg/ml)	Average No. of cells/microcolony	Per cent survival after multiplicity correction
0	4.03	0.48
2.5	4.2	0.45
5.0	4.1	0.46
7.5	3.8	0.47
10.0	3.3	0.44
16.0	2.98	0.30

Table 4

Levels of sialic acid detected in medium from cultures exposed to various concentrations of vitamin A

Vitamin A (μmol/ml)	Time of exposure (hours)	Sialic acid released (μmol)
Ethanol control	24	0.074
15	24	0.139
20	24	0.121
30	24	0.157
40	24	0.146
15	3	0.176
20	3	0.177
30	3	0.160
40	3	0.182

Discussion

The toxic effect reported here for vitamin A on the growth and subsequent plating efficiency of CHO–KI cells supports results obtained by other workers for mouse L-cells, human leukaemia cultures and many other tissues and cells (5, 9, 18, 23). In addition to direct toxic effect, many authors have also reported generalised membrane effects, resulting in cell lysis (17), changes in oxidative phosphorylation and mitochondrial shape and structure (5, 17, 25), various lysosomal effects (2, 6) and cell surface changes (13).

From the results reported here, vitamin A has a complicated effect on the radiation response of CHO–KI cells which depends on dose of both radiation and vitamin A, length of exposure to vitamin A in relation to time of irradiation and cell number exposed. A radioprotective effect predominates at low levels of vitamin A and low or high levels of irradiation. It is also seen with high levels of vitamin A if the level of irradiation is low. A reduction in relative survival becomes prominent where both the level of vitamin A and the radiation dose are high. A radiosensitising effect has been described before (4) for mouse L-cells but experiments were only carried out in detail at one concentration of the vitamin and therefore do not confirm or contradict the data presented here. A radioprotective effect does not appear to have been reported previously.

The finding of an altered radiation response in cells treated with vitamin A is interesting since similar alterations in the function of membrane bound organelles affected by vitamin A have been reported following radiation treatment in the dose range used here (3, 15, 19, 21, 32). This may indicate the involvement of a common membrane associated process in the mechanism of action of vitamin A and of radiation. It is interesting that recovery experiments using cells pretreated with the vitamin showed a reduction in both the rate and level of recovery in treated cells because many other substances, which in common with vitamin A have a pharmacologic site of action associated with the lipid fraction of cellular membranes, are also radiobiologic modifiers, and these also alter the ability of cells to repair or recover from radiation injury. Examples include chloropromazine (28), procaine (11), vitamin E (10). This again could suggest the involvement of membrane associated phenomena at least in the final expression of radiation damage.

The results in Fig. 5 indicate that where low vitamin A levels are used a long period of pre-incubation with vitamin A is required before a radioprotective response can be detected in the cells. Under these conditions vitamin A could be influencing the repair of potentially lethal damage (PLD), for example through cell cycle related mechanisms. Haddox & Russell (11) have shown that vitamin A can reduce a specific growth inhibition block. Alternatively, PLD repair could be affected through stimulation of DNA repair enzymes since there is evidence that retinoids can influence protein synthesis through the activity of retinol binding protein in the nucleus (16, 30). A mechanism involving PLD repair is also suggested by the fact that the protective effect is highly dependent on cell number (Table 2) since cell density and cell-cell contact are important factors for the repair of potentially lethal lesions (31). However, it is likely that other mechanisms are implicated in the effect, particularly in view of the data in Fig. 4 showing that the radiation dose and the vitamin A level together determine the response. This could lend support to a mechanism involving membrane stability; vitamin A is known to stabilise membranes up to a certain level and then destabilise them (24). The data in Table 4 showing that the sialic acid levels in medium from samples of the cells used for these experiments are about twice the control level support this. Sialic acid release indicates disturbance to cell surface receptors and these facts, coupled with the evidence in the literature cited above for the involvement of membranes in the final expression of radiation damage, could suggest a single concentration dependent synergistic mechanism involving membrane stability, where increasing levels of vitamin A lead first to a relative increase in ability to tolerate a cellular insult, possibly followed by destabilisation of cellular defence mechanisms.

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